Accordingly Applicants file simultaneously herewith a Substitute Specification, as well as a redlined version of the original specification, showing the changes make in the Substitute Specification. The changes made in the Substitute specification do not constitute new matter, and thus entry is requested.

In paragraph 3, on page 2 of the Office Action, the Examiner rejects Claim 10 under 35 U.S.C. § 112, first paragraph as containing new matter.

Specifically, the Examiner states that Claim 10 recites the limitation "in the range of 6.5 Gy to 7.0 Gy". The Examiner notes Applicants' assertion that support for this limitation can be found at page 14, lines 7-16 of the present specification. However, the Examiner contends that this disclosure recites only doses of 6.5 and 7.0 Gy, not a range of 6.5 to 7.0. That is, the Examiner states that the specification, at page 14, lines 9-11 recites "at least 6.5 Gy, and yet sublethal dose, preferably about 7.0 Gy". The Examiner contends that reciting "at least 6.5" and "about 7.0" does not support reciting a range of 6.5 to 7.0.

For the following reasons, Applicants respectfully traverse the Examiner's rejection.

Applicants did not assert that support for the recited range can be found at page 14 of the specification. Rather, Applicants asserted that support can be found at page 29 of the specification (see page 3 of the Amendment filed May 1, 2001).

Applicants submit that page 29 of the specification provides support for the range of 6.5 Gy to 7.0 Gy. That is, in the experiment described therein, total body irradiation was

performed at doses of 6.5 Gy and 7.0 Gy. The results, which are discussed at page 30 of the present specification and are shown in Figure 2, demonstrate that mice given an irradiation dose of 7.0 Gy showed an engraftment rate of 100% after transplantation and 3 out of the 3 mice dosed at 6.5 Gy in the portal administration group had successful engraftment.

It is well-settled law that one can claim a range based upon the specific examples provided in the specification (*Ex parte* Jackson, 110 USPQ 561 (Bd. Pat App. & Int. (1956)), wherein the examples showed using 4%, 15%, 20% and Applicants claimed a range 4-20%).

Accordingly, Applicants submit that the specification clearly provides support for a range of 6.5 to 7.0 Gy as recited in Claim 10. Thus, Applicants respectfully submit that the Examiner's rejection is improper and must be withdrawn.

In paragraph 4, on page 3 of the Office Action, the Examiner rejects Claims 9-12 under 35 U.S.C. § 112, first paragraph.

Specifically, the Examiner states that while the specification is enabling for inducing immunotolerance in mice, such is not enabling for inducing immunotolerance in larger organ transplant recipients, such as humans. The Examiner contends that the dose effective in mice would not be effective in humans because humans have a great deal more body mass than mice.

For the following reasons, Applicants respectfully traverse the Examiner's rejection.

The Examiner is requested to note that the "dose" set forth in these claims is an "absorbed dose". That is, the unit of

dose recited in the claims is gray (abbreviated Gy), which represents the absorption of an average of one joule of energy per kilogram of mass in the target material. When a recipient is subjected to total body irradiation (TBI) as claimed in the present application, each cell of the recipient receives substantially the same absorbed dose of radiation regardless of the body weight of the recipient.

Moreover, bone marrow cells of mice and humans may have a slight difference in their sensitivities to radiation. However, they are fundamentally the same. Therefore, they receive the same level of damage from a specific "absorbed dose".

Hence, the basis of the Examiner's rejection, i.e., "the dose effective in mice would not be effective in humans because humans have a great deal more body mass than mice" is technically improper because the claims refer to an "absorbed dose", i.e., Gy, not a dose *per se*.

The Examiner also states that the specification fails to disclose any type of measurable property or process induced in the mouse, such as enhancement or inhibition of cell proliferation or changes in cytokine levels as examples, other than the end result of enhanced graft tolerance. The Examiner contends that without such a measurable property which can be correlated to a level of sublethal irradiation in humans, an undue level of trial and error experimentation would be required.

As explained above, one skilled in the art knows that the level of sublethal irradiation has the same effect on humans and mice.

recited in the claims is gray (abbreviated Gy), which represents the absorption of an average of one joule of energy per kilogram of mass in the target material. When a recipient is subjected to total body irradiation (TBI) as claimed in the present application, each cell of the recipient receives substantially the same absorbed dose of radiation regardless of the body weight of the recipient.

Moreover, bone marrow cells of mice and humans may have a slight difference in their sensitivities to radiation. However, they are fundamentally the same. Therefore, they receive the same level of damage from a specific "absorbed dose".

Hence, the basis of the Examiner's rejection, i.e., "the dose effective in mice would not be effective in humans because humans have a great deal more body mass than mice" is technically improper because the claims refer to an "absorbed dose", i.e., Gy, not a dose *per se*.

The Examiner also states that the specification fails to disclose any type of measurable property or process induced in the mouse, such as enhancement or inhibition of cell proliferation or changes in cytokine levels as examples, other than the end result of enhanced graft tolerance. The Examiner contends that without such a measurable property which can be correlated to a level of sublethal irradiation in humans, an undue level of trial and error experimentation would be required.

As explained above, one skilled in the art knows that the level of sublethal irradiation has the same effect on humans and mice.

Moreover, Fig. 2 and the description on page 30 of the present specification show that the object of the present invention, i.e., successful engraftment of the transplanted cells (an engraftment rate of 100% after transplantation) is achieved. Therefore, it would be apparent to one skilled artisan that inducing immunotolerance, i.e., successful engraftment of the transplanted cells, in humans can be conducted using the sublethal radiation doses described.

Furthermore, enhancement of inhibition of cell proliferation or changes in cytokine levels, referred to by the Examiner, are measurements of the level of a engraftment. Therefore, it is unnecessary to measure the same when the engraftment rate is 100%, which is achieved in the present invention.

In addition, the present invention uses graft donor-specific organs or bone marrow cells, which function normally. Hence when engraftment thereof is conducted successfully, the organs and cells naturally fulfill their original functions. Thus, there is no need to measure enhancement or inhibition of cell proliferation or changes in cytokine levels, as apparently contended by the Examiner.

In any event, Applicants respectfully submit that only routine trial and error experimentation would be required, since the claims specifically recite the minimal amount of radiation which would be effective. The Examiner has failed to provide any evidence that a dose of 6.5 Gy would not be effective in mammals other than mice.

Accordingly, Applicants respectfully submit that the claims are enabled by the present specification, and thus request withdrawal of the Examiner's rejection.

In view of the submission of the Substitute Specification and the arguments set forth above, reexamination, reconsideration and allowance are respectfully requested.

The Examiner is invited to contact the undersigned at the telephone number listed below on any questions that might arise.

Respectfully submitted,

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Date: November 16, 2001

SUBSTITUTE SPECIFICATION - USSN 09/380,579
(MARKED-UP VERSION)

IMMUNOTOLERANCE INDUCER

This application is a §371 of PCT/JP98/00909, filed March 4, 1998.

TECHNICAL FIELD

This invention relates to an immunotolerance inducer ~~capable of~~ for use in organ transplantation, and more particularly to an immunotolerance inducer capable of inducing the immunological tolerance necessary for the maintenance of transplanted organs.

BACKGROUND ART

~~The~~ An immunosuppressant is an indispensable adjunct to organ transplantation, and novel immunosuppressants are constantly being developed ~~one after another~~. According to ~~the~~ their intended ~~application (use)~~, immunosuppressants can be classified into the following two categories. (1) Drugs, ~~of one category are those~~ which are taken daily as long as the graft remains in the recipient's body, for the prophylactic suppression of graft rejection. These drugs ~~and~~ are variously ~~called~~ known as maintenance immunosuppressants, prophylactic immunosuppressants, and basal immunosuppressants. (2) Drugs, ~~of the other category are those used~~which are taken in massive doses, though for limited periods of time, ~~with an aim to causing an intensive~~ which cause immunosuppression ~~necessary for the~~sufficient to

cure ~~of~~ the rejection response, chiefly cellular rejection, which may occur notwithstanding sustained immunosuppression~~, chiefly cellular rejection, and~~. These drugs are known as therapeutic drugs for graft rejection.

However, in terms of ~~the~~ their principal pharmacologic action, as well as side effects, ~~those~~ immunosuppressants can hardly be considered harmless to the human body. ~~, and since~~This is because long-term maintenance doses and/or high doses are required. Thus, toxic effects and/or adverse drug reactions, which cannot be disregarded, are inevitable. Furthermore, ~~those immunosuppressants,~~ when used independently, immunosuppressants are not potent enough to produce sufficient immunosuppression, nor are they capable of curing graft rejections at a high cure rate after onset ~~at a high cure rate~~.

Meanwhile, there are sporadic clinical reports~~, though sporadically,~~ on ~~the success with which~~ successfully maintaining grafts ~~were maintained~~ even without administration of an immunosuppressant. These~~, and those~~ favorable ~~outcomes~~ reports have been attributed to the induction of immunological tolerance. If such immunotolerance could be ~~actually established~~maintained, administration of immunosuppressants would not be necessary. Therefore, the artificial induction of immunological tolerance is regarded as a supreme objective in organ transplantation today. The ~~and~~ results of many relevant studies have been reported.

For example, on~~On~~ the methodology for artificial induction of immunological tolerance, reference may be made to: ~~the following report, among others.~~

~~——~~Induction of Tolerance by Transfer of a Splenocyte or Myelocyte Tolerogen in Combination with Administration of an Antimitotic Drug, ~~[~~(Fukuoka, *Acta Med.*, 81(1):~~,~~ 20-40 (1990); and *Microbiol. Immunol.*, 32(3):~~,~~ 283-292 (1988)~~, etc.]~~).

Therein, ~~As~~ the antimitotic drugs~~,~~ 6-mercaptopurine, methotrexate, cyclophosphamide (CP), 5-fluorouracil, azathioprine (AZP) and procarbazine are mentioned. Further, ~~and~~ it is ~~warned~~ reported that cyclosporin A (CsA) and steroids, which are remote, in terms of the mode of action, from ~~those~~ the above-listed antimitotic agents ~~in the mode of action~~, are not suitable for the induction of immunological tolerance.

Hayakawa et al~~.~~ reported their attempt to induce a donor-specific immunocompromised state with FK506 ~~[~~(Keio, *Medicine*, 72(3):~~,~~ 163-176 (1995)~~]~~). Similarly, Muramatsu et al~~.~~ reported on the possibility of inducing immunological tolerance with 15-DSG ~~[~~(Abstract of Papers read before the 20th Congress of the Japan Society of Microsurgery, pages 89-90 (1994))~~]~~.

The present inventors reported previously that~~,~~ ~~in mice,~~ administration of bone marrow cells (particularly hematopoietic stem cells) ~~into~~ to mice, either through the portal vein or by the usual intravenous route, results in entrapment of the

donor-derived cells in the recipient's liver, the establishment of chimerism and induction of immunotolerance ~~(~~*Eur. J. Immunol.*, 24~~,~~:~~—~~1558 (1994)~~)~~.

The object of ~~this~~ the present invention is to provide a ~~technology by which the necessary~~ method whereby immunological tolerance necessary for organ transplantation can be successfully established. Stated differently, an object of this invention ~~has for its object~~ is to provide a novel method for ensuring a positive ~~sustenance~~ maintenance of grafts without use of immunosuppressants, (e.g., ~~for maintenance (by~~ long-term administration of an immuno-suppressant) and, hence, without risks ~~for~~ of serious side effects.

After an intensive study the inventors found that the pharmaceutical regimen described hereinafter meets the above object and have perfected ~~this~~ the instant invention.

DISCLOSURE OF THE INVENTION

The invention provides an immunotolerance inducer for inducing immunological tolerance in a patient undergoing an organ transplantation comprising: a first pharmaceutical composition for portal administration which comprises an effective amount of a tolerogen containing hematopoietic stem cells, hematopoietic progenitor cells, mature lymphocytes or a mixture thereof in combination with a pharmaceutical carrier, and a second pharmaceutical composition for intravenous

administration which comprises an effective amount of said tolerogen in combination with a pharmaceutically-acceptable carrier, and more particularly wherein said tolergen, as an ~~to said~~ immunotolerance inducer ~~comprising~~ comprises a bone marrow cell fraction ~~as said tolerogen~~.

Furthermore, the present invention provides an immunotolerance inducer for ~~application~~ administration to a patient undergoing an organ transplantation, in association with radiation for inducing immunological tolerance in said patient, comprising an effective amount of a tolerogen containing hematopoietic stem cells, hematopoietic progenitor cells or a mixture thereof in combination with a pharmaceutically-acceptable carrier, and more particularly, wherein said tolerogen as a ~~said~~ immunotolerance inducer, ~~comprising~~ comprises a bone marrow cell fraction ~~as said tolerogen~~.

By using the immunotolerance inducer of this invention, ~~the~~ immunological tolerance meeting the above-mentioned object can be successfully established so that the transplanted organ can be maintained in a satisfactory condition.

As described above, the first-mentioned immunotolerance inducer of this invention essentially comprises said first pharmaceutical composition for portal administration and said second pharmaceutical composition for intravenous administration and as far as this constitution is ~~retained~~administered, the pharmaceutical ~~artefact~~

~~and~~ dosage form of each composition ~~are~~ is not particularly restricted.

For example, said first and second pharmaceutical compositions may be provided in a single dosage form or optionally in independent dosage forms. Thus, as typically represented by the examples ~~of use~~ given hereinafter for the immunotolerance inducer of this invention, there is no particular limitation on the type of pharmaceutical ~~artefact and~~ dosage form, as long as it meets ~~only provided that~~ the object of inducing the necessary immunological tolerance ~~is accomplished~~.

The tolerogen containing hematopoietic stem cells, hematopoietic progenitor cells, mature lymphocytes or a mixture thereof, which is the common active ingredient in said first and second pharmaceutical compositions ~~in common~~ may, for example, be a tolerogen derived from the graft donor (an animal of the same strain as the donor). The active ingredient mentioned above may be a bone marrow cell fraction, spleen cell fraction, peripheral blood cell fraction or a fraction comprising a mixture of them, which contains said cells.

The separation and isolation of such tolerogens can be carried out by known procedures. For example, the procedure described by Yamamoto et al. (*Blood*, 88: 445-454 (1996)) and the procedure described in *Protocols in Experimental Cellular Immunity* (Eed. by Mishell et al ~~B. B., Shiigi S.~~

~~M.~~; translated by Katsuyuki et al~~Imai, Susumu Kawaguchi and Takayuki Harada~~, *Rikogaku-Sha*, pages~~pp.~~ 3-12 (~~,~~ 1982)~~)~~ can be employed.

The preferred tolerogen from the graft donor (a human) includes bone marrow cells and peripheral blood cells. The method of harvesting those cells is well known to those skilled in the art. For example, the method for ~~handling~~ harvesting bone marrow cells may be the same as that used in bone marrow transplantation.

The tolerogen for use in the first pharmaceutical composition is preferably a bone marrow cell fraction rich in hematopoietic progenitor cells, a spleen cell or peripheral blood cell fraction containing mature lymphocytes (exclusive of activated lymphocytes) or a mixture thereof. On the other hand, the tolerogen for the second pharmaceutical composition is preferably said bone marrow cell fraction. As the active component of said first and second pharmaceutical compositions, a bone marrow cell fraction is preferred, as mentioned above, but the peripheral blood cell fraction containing the hematopoietic stem cells mobilized from the bone marrow by cytokines, such as G-CSF, is also preferred partly because it contains both ~~of~~ mature lymphocytes and hematopoietic progenitor, cells and partly because such a cell fraction is readily available.

To provide each of said first and second pharmaceutical compositions, the active component can be formulated into a conventional dosage form

known for pharmaceutically-acceptable products containing cellular fractions of this type. The dosage form can be judiciously selected from among a variety of dosage forms for the present purpose. An injectable dosage form can be mentioned as an example. The pharmaceutical carrier or vehicle which can be used in the manufacture of such dosage forms includes a broad range of pharmaceutically acceptable substances. The method of preparation may also follow ~~the~~ established pharmaceutical procedures. In preparing such dosage forms, the various infusions which are in broad use ~~nowadays~~ can also be employed.

In the practice of this invention, said dosage forms can be prepared extemporaneously, if desired, on the occasion of organ transplantation, using the material obtained from the graft donor.

For administration of the first and second pharmaceutical compositions of this invention, it is essential that the first pharmaceutical composition be administered into the portal vein and the second pharmaceutical composition intravenously. The dosage and timing of administration of each pharmaceutical composition are not particularly restricted, but can be judiciously elected by those skilled in the art, ~~only~~ provided that the necessary immunological tolerance ~~may be~~ is successfully established.

A representative treatment modality comprises administering the first pharmaceutical composition into the portal vein and thereafter administering

the second pharmaceutical composition intravenously. The intravenous administration of the second pharmaceutical composition is preferably carried out at the time when, in the mixed lymphocyte reaction of spleen cells, the reactivity of the host's cells to the donor's alloantigen has decreased to a minimum ~~once~~ (e.g. around the 4th day in mice) and then begins to increase again (around the 5th day in mice).

The recommended dosage of the first pharmaceutical composition which is administered into the portal vein is the minimum dose ($3x10^7$ cells in mice) required to ensure that said reactivity to the donor's alloantigen in the mixed lymphocyte reaction after portal administration becomes minimal (maximum inhibition of the reaction) and plateau out.

The recommended dosage of the second pharmaceutical composition which is administered intravenously is approximately the dose ($3x10^7$ cells in mice) required for reconstructing the host's immune system in the transplantation of the ordinary major histocompatibility complex (MHC)-incompatible bone marrow (after irradiation ~~in~~ with a lethal dose in mice).

The mixed lymphocyte reaction test for spleen cells, referred to above, can be carried out ~~in~~ a the routine manner (~~,~~ *Protocols in Experimental Cellular Immunity*, pages 147-149 (~~,~~ 1982)~~,~~. The total dosage for portal and intravenous administration in humans may be the dose used in ~~the~~

conventional bone marrow transplantation. For example, the dosage in terms of bone marrow cells may be about $3x10^8$ cells/kg or more.

By the portal administration of said first pharmaceutical composition and subsequent intravenous administration of said second pharmaceutical composition according to this invention, the desired immunological tolerance can be induced to ensure a satisfactory maintenance of the transplanted organ.

This invention, therefore, provides a method of inducing immunological tolerance using the specific procedures described above.

The above-mentioned result achieved by the method of this invention is not related to the timing of transplantation of a graft material. Thus, the transplantation procedure can be successfully carried out, whether in parallel with the procedure of this invention or after the establishment of immunological tolerance by the procedure of this invention.

In inducing immunological tolerance in accordance with this invention, various other medical treatments and administration of drugs, which are concomitantly practiced in procedures of this kind, can be practiced in combination with the procedure of the invention, unless the effect of the invention is thereby compromized.

As an example, administration of said immuno-suppressants can also be ~~mentioned~~carried out. The method, dosage, and timing of administration of

immunosuppressants can be judiciously selected by those skilled in the art.

The particularly preferred immunosuppressants includes cyclosporin A and FK506, to mention just a few representative drugs, and the dosage and administration method may be those recommended for the known commercial forms of the products. The particularly preferred mode of administration is to administer an immunosuppressant shortly after administration of the first pharmaceutical composition, once or twice, for example around the 2nd day or around the 2nd and 5th days following portal administration.

Furthermore, the present invention provides an immunotolerance-inducing technology method capable of introducing chimerism with a minimum of invasion and with a high degree of certainty, so as to ensure a long-term sustenance maintenance of the state of immunological tolerance state.

Thus, the present invention provides an immunotolerance inducer to be applied, in association with radiation, to a patient undergoing an organ transplantation for inducing immunological tolerance in the patient, which comprises an effective amount of a tolerogen containing hematopoietic stem cells, hematopoietic progenitor cells or a mixture thereof and a pharmaceutically acceptable carrier.

Even when the portal or intravenous administration of the immunotolerance-inducer of this invention is a single-dose administration, the

immunotolerance meeting the object mentioned hereinbefore can be established as ~~far~~ long as the procedure is followed in combination with radiation, with the result that the transplanted organ can be maintained in a satisfactory condition.

The tolerogen~~-~~ containing hematopoietic stem cells, hematopoietic progenitor cells or a mixture thereof which constitutes the active component ~~of the medicine of~~in this invention for administration to the patient in association with radiation, may, for example, be a bone marrow cell fraction, a peripheral blood cell fraction, or a mixture thereof, which contains hematopoietic stem cells and hematopoietic progenitor cells.

The tolerogen derived from the graft donor (e.g., a human) ~~includes~~ may be a bone marrow cell fraction, an umbilical blood cell fraction ~~and~~ or a peripheral blood cell fraction containing hematopoietic stem cells mobilized by a cytokine, such as G-CSF.

The technique for separation and isolation of said tolerogen for use in the immunotolerance inducer of this invention to be applied in association with said radiation, the method of manufacture of the inducer, its dosage form, the pharmaceutical carrier for use in the manufacture of the dosage form, the route of administration and dosage may all be the same as those mentioned hereinbefore for the first pharmaceutical composition for portal administration and the second

pharmaceutical composition for intravenous administration.

It is essential, however, that the above immunotolerance inducer of this invention be used in association with radiation, that is to say that it be administered into the portal vein or intravenously to the patient given said radiation.

The reference dosage for intravenous administration is roughly the dose ($3x10^7$ cells in mice) required for reconstructing the host's immune system in the transplantation of the ordinary major histocompatibility complex (MHC)-incompatible bone marrow (after irradiation ~~in~~ with a lethal dose in mice).

With the above dosage for mice being taken as a reference, the dosage of the ~~medicine of this~~present invention can be judiciously selected according to the conventions of bone marrow transplantation. As a specific example, the dose of about $3x10^8$ cells/kg or more, in terms of bone marrow cells, can be ~~mentioned~~used.

The radiation mentioned above can be carried out in the conventional manner. More particularly, the patient (recipient) undergoing ~~an~~ organ transplantation is exposed to a suitable radiation dose, for example, an at least 6.5 Gy, and yet sublethal, dose, preferably about 7.0 Gy, per exposure, on a total body irradiation (TBI) basis. This radiation dose is characterized also as a radiation dose providing for recovery of the recipient's bone marrow cells.

The above radiation can be carried out before administration of the ~~medicine~~ immunotolerance inducer of this invention. Preferably, the immunotolerance inducer ~~and usually the medicine~~ is ~~preferably~~ administered within 24 hours of irradiation.

This invention is advantageous in that the expected efficacy can be obtained by a single dose of ~~medication~~ the immunotolerance inducer, which is least invasive to the recipient.

By administering the ~~medicine~~ immunotolerance inducer of this invention in conjunction with radiation, the desired immunological tolerance can be induced for ~~a~~ satisfactory maintenance of the transplanted organ.

The present invention, therefore, provides an immunotolerance~~-~~ ~~-~~inducing method involving radiation.

The phenomenon that the desired immunological tolerance is induced by this method involving radiation for ~~a~~ successful maintenance of ~~the~~ a graft is also unrelated to the timing of the operation for transplantation of the graft.

In addition, in practicing the above combination treatment method, ~~too,~~ the various medical treatments and medications which are usually given in procedures of this kind, for example administration of immunosuppressive drugs such as cyclosporin A, FK506, etc., can be carried out concomitantly unless the effect of the invention is diminished or cancelled.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic representation of the engraftment rate of skin grafts in Test Example 3 ~~and~~.

Fig. 2 is a diagrammatic representation of the engraftment rate of skin grafts in Test Example 4.

BEST MODE FOR CARRYING OUT THE INVENTION

The following is a description of the tests performed with the active component of this invention for illustrating this invention in further detail.

Test Example 1

The induction of immunological tolerance in the test examples was effected by (1) the injection of an allogeneic donor's spleen cells or bone marrow cells into the portal vein and (2) the intravenous injection of the allogeneic donor's bone marrow cells, and the establishment of immunological tolerance was evaluated using, as an indicator, the engraftment rate of skin grafts (allogeneic to the donor) which is the organ most susceptible to rejection ~~as an indicator~~.

(1) Preparation of a spleen cell suspension

Spleen cells were harvested from 8-week-old female BALB/cCrSlc mice (body weights 19-22 g, BALB/c; Japan SLC Inc.) and ~~loosened up~~separated ~~with a pair of non toothed forceps~~ on a 200 G stainless steel mesh screen in RPMI1640 solution

(Nikken Bio Med. Lab.) (with a pair of non-toothed forceps) to prepare discrete spleen cells. The cells were washed with RPMI1640 solution once and subjected to hemolysis with Tris-HCl-ammonium chloride buffer (0.75% NH_4Cl, 0.017 M Tris-HCl, pH 7.5). After two further washings with RPMI1640, the spleen cells were resuspended in the same solution to provide a spleen cell suspension (concentration: $1.0x10^8$/ml).

(2) Preparation of a bone marrow cell suspension

The femurs and tibias were isolated from 8-week-old female BALB/c mice ~~and a~~. A 22-G needle (Code No. NN-2225R, Terumo Co., Ltd.) attached to a syringe (2.5 ml, Code No. SS-02S, Terumo Co., Ltd.) was inserted into each bone from the knee joint side and the bone marrow cells were flushed into a sterilized dish (90x16 mm, Iwaki Clinical Test Ware) using RPMI1640 solution, ~~and suspended~~ followed by suspension in RPMI1640 solution. The bone marrow cells thus harvested were washed with RPMI1640 solution once and resuspended in the same solution to provide the objective bone marrow cell suspension (concentration: $1.0x10^8$/ml).

(3) Injection into the portal vein

Under pentobarbital anesthesia (Pitman-Moor Inc.; 37.5 mg/kg body weight, i.p.), 10-week-old female C57BL/6CrSlc mice (B6; body weights 20-24 g, Japan SLC Inc.) were shaved of hair~~s~~ with a razor and disinfected. Then, a midline incision was made

in the abdominal region and the mesenterium was exposed. A 27 G needle (Terumo Co., Ltd.) attached to a 1 ml-tuberculin syringe was inserted through the adipose tissue of the mesenterium and $3x10^7$ BALB/c mouse spleen cells or bone marrow cells (0.3 ml suspension) prepared in (1) and (2) above were administered into the portal vein.

(4) Intravenous injection

The bone marrow cell suspension prepared in ~~(1)~~ (2) above was adjusted to a concentration of $1x10^8$ cells/ml and ~~the~~ $3x10^7$ cells ~~—~~ equivalent of the suspension (0.3 ml) was administered into the tail vein of the host mouse at day 5 after the portal injection described in (3) above.

(5) Skin grafting

Skin grafting was carried out at day 7 after portal injection. The preparation of skin graft materials and the transplantation thereof were carried out as follows, with reference to the procedure described in the literature (~~†~~Mayumi ~~—~~et al~~.~~, *Jpn. J. Surg.*, 18~~,~~ ~~—~~:548-557 (1988)~~)~~~~†~~.

Thus, as the donor, 8-week-old BALB/c mice were sacrificed under ethyl ether (Nacalai Tesque Inc.) anesthesia. Using a depilatory cream (Feather Hair Remover, Feather Softy Razor Co., Ltd.), the whole hair coat was removed and after disinfection with 70% alcohol, the full-thickness skin layer was peeled off and recovered. After as much subcutaneous adipose tissue was removed as possible,

with a pair of forceps (bent tip, tapered, non-toothed) and sanitary cotton balls ~~as much as possible~~, the skin was cut into a flap (1.2x1.5 cm^2). A 1 mm-long incision was made on the cranial side of the flap as a marker and the flap was suspended in cold sterile phosphate-buffered saline (Dulbecco's PBS(-), Nissui Pharmaceutical Co., Ltd.).

After a B6 host mouse was anesthetized with pentobarbital (37.5 mg/kg body weight, i.p.), the right dorsal region was plucked of hairs with fingers and further depilated with said depilatory cream (3.0x3.5 cm), followed by disinfection ~~and disinfected~~ with 70% alcohol to prepare an operating field for skin grafting.

On the denuded area, the BALB/c skin flap prepared above was placed with the marker disposed caudad and sutured in 8 stitches (in the center of each side and at the 4 corners) using a nylon suture with a 6-0 needle (Ethilon; Ethicon Inc.). The surface of the skin graft was covered with a patch of gauze carrying a fradiomycin sulfate ointment (2.0x2.5 cm, Sofratulle; Japan Roussel Co., Ltd.) and further occluded with an adhesive elastic bandage (Elatex; Alcare Co., Ltd.).

The check for engraftment was started at week 2 after transplantation.

(6) Results

The results are shown in Table 1.

Table 1

	Tolerance procedure		Engraftment of skin graft	
	p.v.	i.v	Time after grafting	Engraftment rate (%)
Test group 1	Spleen Cells	Marrow Cells	36	100 (10/10)
Test group 2	Spleen Cells	Spleen Cells	18	20 (1/5)
Test group 3	Marrow Cells	Marrow Cells	36	67 (4/6)
Control group 1	Spleen Cells	-	3	0 (0/4)
Control group 2	Marrow Cells	-	3	0 (0/4)

(7) Explanation of results and discussion

Test group 1: BALB/c mouse-derived spleen cells were administered into the portal vein of 10 MHC-incompatible B6 mice. At day 5 after administration, BALB/c mouse bone marrow cells were injected intravenously, and at day 7, skin grafting was performed. As a result, engraftment of the transferred skin material was confirmed in 10 of 10 mice at week 36 after transplantation.

Test group 2: BALB/c mouse-derived spleen cells were administered into the portal vein of 5 B6 mice and at day 5 after administration, BALB/c mouse-derived spleen cells were injected intravenously. Skin grafting was performed at day 7. As a result, engraftment was confirmed in 1 of 5 mice at week 18 after transplantation. The

~~but the~~ graft was rejected (dislodged) in one mouse at week 6 and in 3 mice at week 7.

Test group 3: BALB/c mouse bone marrow cells were administered into the portal vein of 6 B6 mice, and at day 5, BALB/c mouse-derived bone marrow cells were administered intravenously. Skin grafting was performed at day 7. As a result, engraftment of the transferred skin was found in 4 of 6 mice at week 36 after transplantation.

Control group: BALB/c mouse-derived spleen cells were administered into the portal vein of 4 B6 mice and skin grafting was performed at day 7. As a result, the graft was rejected (dislodged) in 2 mice at week 2 after transplantation and in the remaining 2 mice at week 3.

Control group 2: BALB/c mouse-derived bone marrow cells were administered into the portal vein of 4 B6 mice and skin grafting was performed at day 7. As a result, the skin graft was rejected in 2 mice at week 2 after transplantation and in the remaining 2 mice at week 3.

It is clear from the above results that the portal administration of the first pharmaceutical composition and subsequent intravenous administration of the second pharmaceutical composition ensure a successful engraftment of the donor's skin graft (maintenance of the donor's alloantigen-specific immunotolerance).

Furthermore, when an immunosuppressant was administered between the portal administration (day 0) and intravenous administration (day 5) of

bone marrow cells in the above test group 3, an improvement was obtained in the engraftment rate of transferred skin grafts. The following test examples will cast more light on the above findings.

Test Example 2

(1) Preparation of bone marrow cells and administration into the portal vein and by the intravenous route

Bone marrow cells were prepared and administered in the same manner as the above Test Example 1-(2), (3) and (4).

(2) Administration of an immunosuppressant

As the immunosuppressant, either cyclosporin A (CsA; Sandimmum, 250 mg/5 ml solution, Novartis Pharma K.K.) 10 mg/kg body weight or FK506 (10 mg/ml solution, Fujisawa Pharmaceutical Co., Ltd.) 1 mg/kg body weight was administered intraperitoneally at day 2 and day 5 after portal administration.

(3) Skin grafting

Skin grafting was performed in the same manner as Test Example 1-(5).

(4) Results

The results are shown below in Table 2.

Table 2

	Tolerance procedure			Engraftment of skin graft	
	p.v.	Immuno-supprresant	i.v	Time (W) after grafting	Engraft-ment rate (%)
Test group 3	Marrow Cells	-	Marrow Cells	36	67 (4/6)
Test group 4	Marrow Cells	CsA	Marrow Cells	32	80 (4/5)
Test group 5	Marrow Cells	FK506	Marrow cells	30	83 (5/6)
Control group 2	Marrow Cells	-		3	0 (0/4)

(5) Explanation of results and discussion

Test group 3 and control group 2 have been fully described in the section of Test Example 1.

Test group 4: BALB/c mouse-derived bone marrow cells were administered into the portal vein of 5 B6 mice. At day 2 and day 5, CsA was administered. In addition, BALB/c mouse-derived bone marrow cells were administered intravenously at day 5 and skin grafting was performed at day 7. As a result, the skin graft was rejected in 1 mouse at week 6 after transplantation. Engraftment ~~but engraftment~~ was thus obtained in 4 of 5 mice, at week 32 after transplantation.

Test group 5: BALB/c mouse-derived bone marrow cells were administered into the portal vein of 6 B6 mice. At day 2 and day 5, FK506 was administered. In addition, BALB/c mouse-derived bone marrow cells were administered intravenously at day 5 and skin

grafting was performed at day 7. As a result, the skin graft was rejected in 1 mouse at week 6 after transplantation. ~~but engraftment~~Engraftment was thus obtained in 5 of 6 mice, at week 30 after transplantation.

The following conclusion can be drawn from the above findings. Although it was generally acknowledged that immunosuppressants such as CsA and FK506 are not suited for use in combination with a tolerogen for the induction of immunotolerance, the use of an immunosuppressant in combination with the portal administration of the first pharmaceutical composition and the intravenous administration of the second pharmaceutical composition in accordance with this invention results in an improved engraftment rate and is, therefore, effective in inducing immunological tolerance.

Thus, in accordance with this invention, there is provided a new immunotolerance inducing technique which can be fully expected to find clinical application.

Test Example 3

(1) Preparation of bone marrow cells and the portal and intravenous injection of the cells

The procedures described in Example 1-(2), (3) and (4) were repeated.

(2) Administration of an immunosuppressant

CsA, 10 mg/kg body weight, was administered intraperitoneally at day 2 and day 5 after portal injection.

(3) Skin grafting

Except that skin grafting was performed on the same day as the portal administration, the procedure of Test Example 1-(5) was repeated herein (n=6). A control group receiving skin flaps derived from C3H mice was also provided (n=4).

(4) Results

The results are shown in Fig. 1.

In Fig. 1, the ordinate represents the engraftment rate (%) of skin flaps and the abscissa represents time (in weeks) after grafting. Group 1 is a test group and Group 2 is a control group.

The results of this test example indicate that the immunotolerance-inducing procedure comprising administration of the first pharmaceutical composition into the portal vein and the organ transplantation can be concurrently carried out. Therefore, in humans, too, the portal administration of the first pharmaceutical composition (bone marrow and other cells) from the donor and the organ transplantation can be concurrently performed. This technique is considered to be an epochal one in that the graft vs. host reaction (GvH reaction) can be prevented even without removal of T cells from the marrow cell fraction and the immunotolerance can be sufficiently maintained using only two doses of an immunosuppressant.

Test Example 4

The induction of immunotolerance was carried out by the portal or intravenous injection of an allogeneic donor's bone marrow cells and the

establishment of immunological tolerance was evaluated using the engraftment rate of the skin grafts (allogeneic to the donor), which are most susceptible to rejection, as an indicator.

(1) Preparation of a bone marrow cell suspension

From ~~the~~ a donor mouse, the femurs and tibias were removed and a 22 G needle (Code No. NN-2225R, Terumo Co., Ltd.) attached to a syringe (2.5 ml, Code No. SS-02S, Terumo Co., Ltd.) was inserted into each bone from the knee-joint side. The bone marrow cells were flushed into a sterilized dish (90x15 mm, Iwaki Clinical Test Ware) using RPMI1640 solution from the syringe and suspended in RPMI1640 solution. The harvested bone marrow cells were washed with RPMI1640 solution once and resuspended in the same solution to provide the objective bone marrow cell suspension (concentration: $1x10^8$ cells/ml).

(2) Radiation

Irradiation of ~~the~~ recipient mice was carried out by the TBI method using Gamma Cell 40 Exacter (Nordion International Inc.) and ^{137}Cs as a beam source.

(3) Portal administration

The recipient mouse was shaved of hair~~s~~ with a razor under pentobarbital anesthesia (Pitman-Moor Inc.; 37.5 mg/kg body weight, i.p.) and after disinfection, a midline incision was made in the abdomen and the mesenterium was exposed. A 27 G needle (Terumo Co., Ltd.) attached to a 1 ml-tuberculin syringe was inserted through the adipose tissue of the mesenterium and $3x10^7$ bone

marrow cells from the donor mouse (0.3 ml of the suspension prepared above) were administered into the portal vein.

(4) Intravenous administration

The bone marrow cell suspension prepared above from the donor mouse was adjusted to $1x10^8$ cells/ml and ~~the~~ a $3x10^7$ cell equivalent thereof (0.3 ml) was administered ~~from~~ into the tail vein of the recipient mouse.

(5) Skin grafting

The preparation and transplantation of skin grafts were carried out as follows, with reference to the procedures described in the literature (~~[~~Mayumi et al~~.~~, *Jpn. J. Surg.*, 18~~,~~~~:~~548-557 (1988)~~]~~.

Thus, ~~the~~ a donor mouse was sacrificed under ethyl ether (Nacalai Tesque Inc.) anesthesia and the whole hair coat was removed with a depilatory cream (Feather Hair Remover, Feather Safety Razor Co. Ltd.). After disinfection with 70% alcohol solution, the full-thickness skin layer was peeled off and recovered. Using a pair of forceps (with a bent tip, tapered, non-toothed) and sanitary cotton balls, as much of the subcutaneous fat tissue was removed ~~as much~~ as possible and the skin was cut into a flap (1.2x1.5 cm square). A 1 mm-incision was made on the cranial side of the flap as a marker and the skin flap was left floating in cold sterilized phosphate-buffered saline (Dulbecco's PBS(-), Nissui Pharmaceutical Co. Ltd.).

After the recipient mouse was anesthetized with pentobarbital (37.5 mg/kg body weight, i.p.), the right dorsal region was plucked of hairs with fingers and further depilated with said depilatory cream (3.0x3.5 cm). The denuded area was disinfected with 70% alcohol solution to prepare an operating field for skin grafting.

On the denuded area, the donor's skin flap prepared above was placed with the marker disposed caudad and sutured in 8 stitches (in the center of each side and at the 4 corners) using a nylon suture with a 6-0 needle (Ethilon; Ethicon Inc.). The surface of the graft was covered with a patch of gauze carrying a fradiomycin ointment (2.0x2.5 cm, Sofratulle; Japan Roussel Co., Ltd.) and further occluded with an adhesive elastic bandage (Elatex; Alcare Co., Ltd.).

(6) Induction of immunological tolerance

Using (BALB/cxDBA2) F1 mice (H-2K^{d}) (aged 7~8 weeks, 19~20 g, Japan SLC) as donor mice and B6 mice (H-2K^{b}) (aged 10~13 weeks, 20~23 g, Japan SLC) as recipient mice, each recipient animal was irradiated and, after 1 day, the donor's bone marrow cells were administered either into the portal vein or intravenously. Skin grafting was performed within the same day as the portal or intravenous administration of bone marrow cells and ~~the check for~~ engraftment of skin flaps was ~~made~~ determined starting week 3 after transplantation.

(7) Results

The results are shown in Fig. 2.

In Fig. 2, the ordinate represents engraftment rate (%) and the abscissa represents time (in weeks) after transplantation. The legend Group I represents the data generated in a group (n=3) which received a radiation dose of 6.5 Gy in association with portal administration of bone marrow cells [Group I: 6.5 Gy+pv (n:3)]; the legend Group II represents a group which received a radiation dose of 7.0 Gy in association with portal administration (n=9) or intravenous administration (n=5) of bone marrow cells [Group II: 7 Gy+pv (n=9) or iv (n=5)]; the legend Group III represents a group which received a radiation dose of 6.5 Gy in association with intravenous administration of bone marrow cells (n=7) [Group III: 6.5 Gy+iv (n=7)]; and the legend Group IV represents a group which received a radiation dose of 6.0 Gy in association with portal administration (n=5) or intravenous administration (n=3) of bone marrow cells [Group IV: 6.0 Gy+pv (n=5) or iv (n=3)].

(8) Explanation of the results

In B6 mice, total body irradiation was performed ~~in~~ with a dose of 7.0 Gy, 6.5 Gy or 6.0 Gy and after about 24 hours, the portal (pv) or intravenous (iv) injection of bone marrow cells derived from a (BALB/cxDBA/2) F1 mouse (CDF1) was carried out. Then, within the same day, skin grafting was performed. As shown in Fig. 2, the recipient mice given a radiation dose of 7 Gy in

both the portal and intravenous administration groups showed an engraftment rate of 100% for the donor (CDF1)'s skin graft at week 23 (on the 167th day) after transplantation (9 of 9 mice in the pv group and 5 of 5 mice in the iv group). This is in contrast with the recipient mice exposed to a radiation dose of 6.0 Gy, in which the skin graft was invariably rejected within 3 weeks after transplantation (5 of 5 mice in the pv group and 3 of 3 mice in the iv group). In the recipient mice given a radiation dose of 6.5 Gy, the skin graft was rejected in one of 7 mice in the intravenous administration group at week 3 after transplantation but successful engraftment was obtained in 3 of the 3 recipient mice in the portal administration group at week 13 after transplantation.

(9) Discussion

The engraftment rate was slightly higher in the 6.5 Gy plus portal administration group. It appears that because the donor's hematopoietic stem cells are trapped in the recipient's liver with higher efficiency in this group, the rejection by radio-resistant immunocompetent cells in the recipient mice is more effectively avoided.

Pharmaceutical Example 1

Bone marrow cells or spleen cells are suspended in physiological saline to prepare a $1x10^8$/ml composition for administration into the portal vein. On the other hand, a composition containing $1x10^8$ bone marrow cells/ml saline is similarly prepared for intravenous administration.

In humans, the above composition for portal administration is preferably administered in a dose of generally $3x10^8$ bone marrow cells or more (T cells may be present) per kg body weight.

Pharmaceutical Example 2

Bone marrow cells are suspended in physiological saline to provide a $1x10^8$/ml suspension. For administration into the portal vein of a patient, for instance, the suspension is preferably administered in a dose of generally $3x10^8$ bone marrow cells or more (a small proportion, i.e., about 2%, of T cells may be present) per kg body weight. Thus, there is provided an injection containing at least the above unit dose. This injectable composition is of value as an immunotolerance inducer to be used in association with radiation.

INDUSTRIAL APPLICABILITY

With the immunotolerance inducer of this invention, ~~an~~ immunological tolerance can be induced in a patient undergoing an organ transplantation and a positive maintenance of the transplanted organ can ~~therefore~~ thereafter be ~~ensured~~assured.